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(54) Title: METHODS FOR SELECTING GERM–LINE COMPETENT CELLS IN CHICKEN EMBRYOS, AND THE USE OF THE CELLS IN THE PRODUCTION OF CHIMERAS

(57) Abstract

Methods are described for selecting germ–line competent cells in stage X (E–G & K) chicken embryos comprising selecting cells from a stage X (E–G & K) chicken embryo which have an epitope expressed by germ–line competent cells associated with their cell surface. The epitope may be EMA–1 or SSEA–1, for example. Methods for producing a chimeric chicken embryo and a chimeric chicken using the selected germ–line competent cells are also described.

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Title: Methods for Selecting Germ-line C mpetent Cells in Chicken Embryos, and the Use of the Cells in the Production f Chimeras

This application claims benefit from United States provisional application serial no. 60/039,488 filed on February 28, 1997.

FIELD OF THE INVENTION

The invention relates to methods for selecting germ-line competent cells, and methods for producing germ-line chimeras in avian species.

BACKGROUND OF THE INVENTION

The poultry industry has traditionally relied on crossbreeding of pure lines to provide chickens, turkeys and other poultry with desirable characteristics. The genetic rearrangement that occurs at each generation usually results in offspring that bear only a small proportion of the attributes of superior individuals in the parental population. Ideally, breeders would like more control over the manipulation of the genome.

There have been many studies to try to manipulate the genome during the embryonic stage of development to introduce favourable characteristics in poultry. Methods have been used to introduce DNA into the pronuclei or newly fertilized ovum but these methods suffer from many disadvantages. The methods are expensive since the avian species must be killed to obtain each ovum or zygote; it is difficult to identify the male and female pronuclei among the supernumary spermatozoa that enter at fertilization; exogenous DNA does not integrate into the genome at a high rate when injected into newly fertilized zygotes; and it is technically difficult to return the manipulated ovum to the oviduct of a fistulated hen, or to maintain it in a surrogate egg. Therefore, more current methods involve the manipulation of blastodermal cells contained in the newly laid egg.

Fertilization of chicken embryos occurs within 15 minutes following ovulation in the infundibulum of the reproduction tract. The embryo, which is situated on the surface of the yolk, develops during the next 18-23 hours as egg formation is completed by the secretion of albumen, membranes and shell around the yolk. The first cell division occurs approximately 5 hours after fertilization as the egg enters the shell gland. During the next 13-18 hours, embryonic divisions continue rapidly to yield an embryo containing 40,000 - 60,000 cells (Eyal-Giladi & Kochav, 1976; Kochav et al., 1980; Watt *et al.*, 1993). When egg formation is completed, the egg is expelled from the shell gland at oviposition. At oviposition, the embryonic structure which contains 40,000-60,000 cells is designated as a stage X (E-G & K) embryo.

When a sample of up to 1000 cells from a population of cells harvested from stage X (E-G & K) embryos is transferred to recipient embryos at the same stage of development, the donor cells contribute to both somatic tissues and th germ-line f the resulting chimera (Petitte et al., 1990; Carsience et al., 1993, Fraser et al., 1993; Thoraval et al., 1994; Kagami et al., 1995; Etches et al., 1996a; Kin et al., 1997). The identification of

cells that contribute t the germ-line and somatic tissues, however, has not been made. The production of somatic and germ-line chimeras after the injection of cells from stage X (E-G & K) embryos may indicate that each cell in a stage X (E-G & K) embryo can contribute to ectoderm, mesoderm, endoderm and the germ-line and, therefore, each cell from a stage X (E-G & K) embryo is pluripotent. However, some indirect evidence suggests that the population of cells in stage X (E-G & K) embryos contains cells with different functional properties. For example, the EMA-1 epitope, which is believed to be expressed uniquely on primordial germ cells which are committed to the germ-line, is expressed on some cells in stage XI (E-G & K) embryo (Urven et al., 1988; Karagenc et al., 1995). The epitope SSEA-1, which is expressed by mouse embryonic stem cells, is expressed by some cells in stage XIII (E-G&K) embryos (Petitte and Karagenc, 1996). Evidence supporting the presence of morphologically unrecognizable primordial germ cells in stage X (E-G&K) chicken embryos may be inferred from the presence of committed primordial germ cells in cultures derived from the central disc, but not the area opaca, of stage X blastoderms (Ginsburg and Eyal-Giladi, 1987). The presence of cells that are destined for the germ-line can also be inferred from the observation that somatic and germ-line chimeras are produced more frequently from cells taken from the central disc rather than the area opaca of stage X (E-G&K) embryos (Petitte et al., 1993). In the quail, the QH-1 epitope, which is believed to be expressed by cells committed to the germ-line in quail, is expressed in embryonic cells at the time of oviposition (Pardanaud et al., 1987). Although these data provide indirect evidence indicating that the precursors of primordial germ cells are present in stage X (E-G&K) embryos, it is not yet clear if (1) these cells are committed to the germ-line, (2) if they retain the ability to enter both the somatic and germ-line lineages and (3) by what means they can be identified.

The production of chimeric avian species such as chickens would be greatly enhanced if the location of germ-line competent cells within stage X (E-G&K) embryos were identified, and if populations of germ-line committed cells could be isolated from the entire population of cells that comprise a stage X (E-G&K) embryo. For example, it would be possible to extirpate the endogenous germ-line competent cells within a recipient embryo to eliminate any contribution to the germ-line except that of the donor embryo. Using current technology, the endogenous contribution is reduced, but not eliminated, by irradiating the recipient embryo (Carsience et al., 1993). Irradiation impedes growth of the recipient embryo for approximately 24 hours while the donor-derived contributions to the chimera proliferate (Carsience et al., 1993). While this approach has proved to be useful, the extent of the donor-derived contribution to the germ-line is neither predictable nor consistent. The lack of a predictable and consistent contribution to the germ-line is particularly important when the d nor-cell population contains a small number of genetically unique and rare donor cells. Examples of rare cells might include genetically

modified cells (see Brazolot et al., 1991; Fraser et al., 1993) or cells derived from cryopreserved stocks that are extinct (see Kino et al., 1997).

The production of chimeric chickens would also be enhanced if cell surface epitopes expressed by germ-line competent cells within stage X (E-G&K) embryos were identified. The identification of the epitopes would facilitate development of methods to identify and isolate germ-line competent cells.

SUMMARY OF THE INVENTION

The present inventor has identified cell surface epitopes expressed by germ-line competent cells within stage X(E-G&K) embryos of chickens. In particular, the present inventor has identified the EMA-1 and SSEA-1 epitopes in stage X (E-G & K) chicken embryos and in cells derived from them in culture. The identification of the epitopes facilitated the identification and isolation of cells within stage X (E-G&K) chicken embryos that are capable of replication without differentiation *in vitro* and which retain the ability to enter the germ-line following injection into a recipient embryo. These cells are referred to herein as "germ-line competent cells". Isolated germ-line competent cells from donor embryos were introduced into recipient embryos to produce chimeric embryos. The extent of chimerism was increased when donor cells were injected into recipients from which cells had been extirpated from the central region of the recipient embryo and when recipient embryos are irradiated prior to the injection of the donor cells.

Broadly stated the present invention relates to a method for selecting germ-line competent cells in stage X (E-G&K) chicken embryos comprising separating from a stage X (E-G&K) chicken embryo cells that have an epitope expressed by germ-line competent cells associated with their cell surface. In particular, germ-line competent cells may be isolated by reacting cells obtained from a stage X (E-G&K) chicken embryo with a substance which binds directly or indirectly to an epitope expressed by germ-line competent cells. In a preferred embodiment, the epitope expressed by germ-line competent cells is an EMA-1 or an SSEA-1 epitope.

The invention also relates to a method for producing a cell preparation enriched for germ-line competent cells of chickens comprising (a) providing cells from a stage X (E-G&K) chicken embryo; and (b) selecting cells that have an epitope expressed by germ-line competent cells associated with their cell surface.

Germ-line competent cells identified and isolated using the methods of the invention may be genetically modified by introducing a recombinant expression vector into the cells. Therefore, the methods of the invention may additionally comprise transfecting the germ-line competent cells with a recombinant expression vector containing an exogenous gene and the necessary elements for the transcription and translation of the gene.

The invention additionally relates to a cell culture comprising germ-line competent cells or genetically modified germ-line cells obtained using the methods of the invention.

The invention still further relates to a method for identifying a region in an embryo which contains germ-line competent cells comprising

(a) treating the embryo with a substance which directly or indirectly binds to an epitope expressed by germ-line competent cells wherein the substance is labelled with a detectable marker; and

(b) detecting the detectable change produced by the detectable marker to identify a region in the embryo which contains germ-line competent cells.

In a preferred embodiment, the epitope expressed by germ-line competent cells is an EMA-1 or an SSEA-1 epitope and the substance that binds to the epitope is an antibody.

The germ-line cells identified in accordance with the methods of the invention may be used to produce chimeric chicken embryos. Therefore, the present invention provides a method for producing a chimeric chicken embryo comprising (a) isolating from a donor stage X (E-G&K) chicken embryo cells that have an epitope expressed by germ-line competent cells associated with their cell surface; (b) optionally transfecting the cells with a recombinant expression vector containing an exogenous gene and the necessary elements for the transcription and translation of the gene; (c) introducing the cells into a recipient stage X (E-G&K) chicken embryo from which a portion of the central disk of the embryo has been removed; and (d) incubating the recipient embryo to produce a chimeric embryo. The chimeric embryo may be grown to term to produce chimeric chickens.

The invention further provides chimeric embryos and chimeric chickens produced by the methods of the invention.

Still further the present invention relates to kits for performing the methods of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the structure of a stage X embryo illustrated as a cross-section through the embryo perpendicular t the surface of th y lk;

Figure 2 shows a phase contrast illumination micrograph illustrating the expression of EMA-1 epitopes on stage X blastodermal cells.

Figure 3 shows a fluorescence micrograph of Figure 2;

Figure 4 shows a phase contrast illumination micrograph illustrating the expression of SSEA-1 epitopes on stage X blastodermal cells;

Figure 5 shows a fluorescence micrograph of Figure 4;

Figure 6 shows a phase contrast illumination micrograph illustrating the SSEA-1 expression on stage X whole mount;

Figure 7 shows a fluorescence micrograph of Figure 6;

Figure 8 shows a phase contrast illumination micrograph illustrating HNK-1/NC-1 expression on stage X blastodermal cells; and

Figure 9 shows a fluorescent micrograph of Figure 8.

DETAILED DESCRIPTION OF THE INVENTION

I. Selection of Germ-line Competent Cells

As hereinbefore mentioned, the present invention relates to a method for selecting germ-line competent cells in stage X (E-G&K) chicken embryos comprising separating from a stage X (E-G&K) chicken embryo, cells which have an epitope expressed by germ-line competent cells associated with their cell surface. The invention also relates to a method for producing a cell preparation enriched for germ-line competent cells of chickens comprising (a) providing cells from a stage X (E-G&K) chicken embryo; and (b) selecting cells that have an epitope expressed by germ-line competent cells associated with their cell surface. In a preferred embodiment, the epitope expressed by germ-line competent cells is an EMA-1 or an SSEA-1 epitope.

A stage X (E-G&K) chicken embryo is an embryo which is expelled from the shell gland at oviposition and it is characterized by containing between 40,000 - 60,000 cells (Eyal-Giladi & Kochav, 1976; Kochav et al., 1980; Watt *et al.*, 1993). The architecture of a stage X (E-G & K) embryo is illustrated in Figure 1. Morphologically, none of the cells in a stage X embryo demonstrate the characteristics of differentiated cells. The central disc, which includes the area pellucida and the marginal zone, is surrounded by the area opaca. The area pellucida is situated above a layer of subgerminal fluid that separates the embryo from the underlying yolk. The area opaca is directly in contact with the surrounding yolk.

Stage X (E-G&K) embryos may be isolated from eggs using conventional methods, For example, embryos may be isolated from freshly laid unincubated eggs by separating albumen from the yolk as described by Carsience et al (1993)

Prior to selecti n of the germ-line competent cells, cells from a stage X (E-G&K) chicken embryo are preferably dissociated from the embryo using conventional

techniques For example, blastoderm cells may be dispersed from an embryo by treating with a substance which digests the extracellular matrix such as trypsin.

Cells that have epitopes expressed by germ-line competent cells associated with their surface may be selected using substances which directly or indirectly bind to the epitopes. In an embodiment of the invention, the epitope is EMA-1 or SSEA-1 and the substance is an antibody specific for a EMA-1 or SSEA-1 epitope. Therefore, the invention provides a method for selecting germ-line competent cells in stage X chicken embryos comprising reacting cells obtained from a stage X (E-G &K) chicken embryo with one or more antibodies to an epitope expressed by germ-line competent cells; forming conjugates between the antibodies and the germ-line competent cells having the epitope associated with their cell surface; and isolating the conjugates to obtain a cell preparation containing germ-line competent cells. The method may employ more than one antibody, for example one antibody to EMA-1 and one antibody to SSEA-1.

The term "antibody" includes polyclonal antisera or monoclonal antibodies. Conventional methods can be used to prepare the antibodies. For example, by using a EMA-1 and SSEA-1 epitope, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of a EMA-1 or SSEA-1 epitope which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on an epitope include conjugation to carriers or other techniques well known in the art. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, [e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)]. Other techniques such as screening of combinatorial antibody libraries can be employed (Huse et al., Science 246, 1275 (1989)]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the epitopes and the monoclonal antibodies can be isolated.

The term "antibody" also includes antibody fragments which also specifically react with an epitope expressed by germ-line competent cells. Antibodies can be fragmented using conventional techniques and the fragments screened for utility as described above. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment may be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-avian variable region and an avian constant region are also within the scope of the invention. Chimeric antibody molecules include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with avian constant regions. Standard methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes an epitope expressed by germ-line competent cells (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B).

Similarly, binding partners may be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. The primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP™ H or ImmunoZAP™ L (Stratacyte), respectively. These vectors may then be introduced into E. coli for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (See Bird et al., Science 242:423-426, 1988).

Antibodies against an epitope may also be obtained from commercial sources. For example, monoclonal antibodies to SSEA-1 and EMA-1 may be obtained from Immunotech, Westbrook, ME, USA.

The antibodies may be labelled with a detectable marker including various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, ß-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include S-35, Cu-64, Ga-67, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I131, Re-186, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. The antibodies may also be labelled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-biotin and riboflavin-riboflavin binding protein. Methods for conjugating or labelling the antibodies with the representativ labels set forth above may be readily accomplished using conventional techniques.

The antibodies may be labelled with a detectable marker as described herein or indirect methods may be employed. In an indirect method, the primary antigen-antibody reaction may be amplified by the introduction of a second antibody, having specificity for the antibody reactive against an epitope. By way of example, if the antibody having specificity against an EMA-1 or SSEA-1 epitope is a mouse IgG antibody, the second antibody may be rat or rabbit anti-mouse gamma-globulin labelled with a detectable marker as described herein.

The antibodies may be bound to a carrier such as agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The antibodies may be conjugated to a matrix. Examples of matrices are magnetic beads, which allow for direct magnetic separation (Kemshead 1992), panning surfaces e.g. plates, (Lebkowski, J.S, et al., (1994), J. of Cellular Biochemistry supple. 18b:58), dense particles for density centrifugation (Van Vlasselaer, P., Density Adjusted Cell Sorting (DACS), A Novel Method to Remove Tumor Cells From Peripheral Blood and Bone Marrow StemCell Transplants. (1995) 3rd International Symposium on Recent Advances in Hematopoietic Stem Cell Transplantation-Clinical Progress, New Technologies and Gene Therapy, San Diego, CA), adsorption columns (Berenson et al. 1986, Journal of Immunological Methods 91:11-19.), and adsorption membranes (Norton et al. 1994). The antibodies may also be joined to a cytotoxic agent such as complement or a cytotoxin, to lyse or kill the targeted germ-line competent cells.

The antibodies may be directly coupled to a matrix. For example, the antibodies may be chemically bound to the surface of magnetic particles for example, using cyanogen bromide. When the magnetic particles are reacted with a sample containing germ-line competent cells having an epitope expressed by germ-line competent cells on the their cell surfaces, conjugates will form between the magnetic particles with bound antibodies and the germ-line competent cells.

Alternatively, the antibodies may be indirectly conjugated to a matrix using antibodies. For example, a matrix may be coated with a second antibody having specificity for the antibodies to a SSEA-1 or an EMA-1 epitope. By way of example, if the antibodies to the SSEA-1 or EMA-1 epitope are mouse IgG antibodies, the second antibody may be rabbit anti-mouse IgG. The antibodies may also be incorporated in antibody reagents which indirectly conjugate to a matrix. Examples of antibody reagents are bispecific antibodies, tetrameric antibody complexes, and biotinylated antibodi s.

Bispecific antibodies contain a variable region of an antibody specific for an epitope expressed by germ-line competent cells, and a variable region specific for at least

one antigen on the surface of a matrix. The bispecific antibodies may be prepared by forming hybrid hybridomas. The hybrid hybridomas may be prepared using the procedures known in the art such as those disclosed in Staerz & Bevan, (1986, PNAS (USA) 83: 1453) and Staerz & Bevan, (1986, Immunology Today, 7:241). Bispecific antibodies may also be constructed by chemical means using procedures such as those described by Staerz et al., (1985, Nature, 314:628) and Perez et al., (1985 Nature 316:354), or by expression of recombinant immunoglobulin gene constructs.

A tetrameric immunological complex may be prepared by mixing a first monoclonal antibody which is capable of binding to at least one antigen on the surface of a matrix, and a second monoclonal antibody specific for an epitope expressed by germ-line competent cells. The first and second monoclonal antibody are from a first animal species. The first and second antibody are reacted with an about equimolar amount of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. The first and second antibody may also be reacted with an about equimolar amount of the $F(ab')_2$ fragments of monoclonal antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species. (See U.S. Patent No. 4,868,109 to Lansdorp, which is incorporated herein by reference for a description of tetrameric antibody complexes and methods for preparing same).

The antibodies may be biotinylated and indirectly conjugated to a matrix which is labelled with (strept) avidin. For example, biotinylated antibodies may be used in combination with magnetic iron-dextran particles that are covalently labelled with (strept) avidin (Miltenyi, S. et al., Cytometry 11:231, 1990). Many alternative indirect ways to specifically cross-link the antibodies and matrices would also be apparent to those skilled in the art.

In a preferred embodiment of the invention, the cell-antibody conjugates are removed by magnetic separation using magnetic particles. Suitable magnetic particles include particles in ferrofluids and other colloidal magnetic solutions. Examples of ferrofluids and methods for preparing them are described by Kemshead J.T. (1992) in J. Hematotherapy, 1:35-44, at pages 36 to 39, and Ziolo et al. Science (1994) 257:219 which are incorporated herein by reference. Colloidal particles of dextran-iron complex may be used in the process of the invention. (See Molday, R.S. and McKenzie, L.L. FEBS Lett. 170:232, 1984; Miltenyi et al., Cytometry 11:231, 1990; and Molday, R.S. and MacKenzie, D., J.Immunol. Methods 52:353, 1982; Thomas et al., J. Hematother. 2:297 (1993); and U.S. Patent No. 4,452,733, which are each incorporated herein by reference). Magnetic particles may als be obtained from commercial s urces such as th microbeads available from Mitenyi Biotec.

In accordance with a magnetic separation method of the invention, a sample containing g rm-line competent cells to be recovered, is reacted with one of the above described antibody reagents so that the antibody reagents bind to the targeted germ-line competent cells present in the sample to form cell conjugates of the targeted cells and the antibody reagents. The reaction conditions are selected to provide the desired level of binding between the targeted cells and the antibody reagents. The concentration of the antibody reagents is selected depending on the estimated concentration of the targeted cells in the sample. The magnetic particles are then added and the mixture is incubated at the selected temperature. The sample is then ready to be separated over a magnetic filter device. Preferably, the magnetic separation procedure is carried out using the magnetic filter and methods described in Miltenyi et al, 1990. Commercial magnetic separation systems such as the MiniMACS system available from Miltenyi Biotch may also be used in the magnetic separation methods of the present invention.

The sample containing the magnetically labelled cell conjugates is passed through the magnetic filter in the presence of a magnetic field. The magnetically labelled conjugates are retained in the high gradient magnetic column and the materials which are not magnetically labelled flow through the column after washing with a buffer.

Antibodies to an epitope expressed by germ-line competent cells may also be used to detect and quantify germ-line competent cells in an embryo. In particular, the antibodies may be used in immuno-histochemical analyses to localise germ-line competent cells to particular regions in the embryo.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect germ-line competent cells. Generally, an antibody labelled with a detectable marker can be used to localize the germ-line competent cells in an embryo based upon the presence of the detectable marker. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

The reagents suitable for applying the methods of the invention may be packaged into convenient kits providing the necessary materials, packaged into suitable containers. Such kits may include all the reagents required to select a germ-line competent cell in a sample by means of the methods described herein, and optionally suitable supports useful in performing the methods of the invention.

II. Modification of Germ-line Competent Cells

Germ-line competent cells isolated from stage X (E-G&K) embryos in accordance with the methods of the invention may be genetically modified by random or site-directed integration of DNA into the cells. Therefore, the present invention relates t the genetic modification of germ-line competent cells isolated by the methods of the invention comprising introducing a recombinant expression vector containing an exogenous

gene and the necessary elements for the transcription and translation of the gene. Examples of exogenous genes which can be introduced into the germ-line competent cells include vasoactive intestinal peptide, growth hormone, insulin-like growth factor I, the IGF-I receptor, the GH receptor, prolactin, the gonadotrophins, and gonadotrophin-releasing hormone (Etches et al, 1993 and references therein). Selection of the necessary elements for the transcription and translation of the genes may be readily accomplished by one of ordinary skill in the art. The necessary regulatory sequences may be supplied by the native genes and/or their flanking regions.

Transfer of DNA into the germ-line cells may be accomplished using retroviral vectors (Shuman, Experientia, 47:897-904, 1991; Petropoulos et al., Journal of Virology, 66:3391-3397, 1992; Salter et al., Manipulation of the Avian Genome, pp. 135-150, 1993). Large DNA sequences (greater than 2000 base pairs), including those designed to promote specific integration within the genome via homologous recombination, can be introduced by liposome-mediated gene transfer.

Genetically modified germ-line competent cells can be cultured *in vitro* to provide a population of genetically modified germ-line competent cells. Culture systems which have been designed to support growth of unknown types of cells derived from stage X embryos (see Pain et al., 1996) can be used to support the growth of transfected (and untransfected) germ-line competent cells. *In vitro* culture techniques can also be employed to facilitate selection of transfected germ-line competent cells. For example, the techniques utilized to select transfected cells from non-transfected embryonic stem cells in mice (Mansour et al., Nature, 366:248-252, 1988) may be used in the present invention.

Single germ-line competent cells may also be cloned using conventional techniques to provide a homogenous population of genetically modified donor cells.

III. Production of Germ-line Chimeras

The germ-line competent cells selected using the methods of the invention may be used to produce chimeric chicken embryos. Chimeric chicken embryos are produced by isolating from a donor stage X (E-G&K) chicken embryo using the methods described herein, germ-line competent cells that have an epitope expressed by germ-line competent cells associated with their cell surface. In a preferred embodiment the epitope is EMA-1 or SSEA-1. The isolated germ-line competent cells may be transfected with a recombinant expression vector containing an exogenous gene and the necessary elements for the transcription and translation of the gene as described herein.

The germ-line competent cells are introduced into a recipient stage X (E-G&K) chicken embryo from which a portion of the central disk of the embryo has been removed. The cells are introduced using conventional methods such as injection into the subgerminal cavity of the recipient embryo. A portion of the central disk of th recipient embryo is removed using physical techniques, or using antibodies to an epitope conjugated to a t xin or

to complement to kill recipient germ-line competent cells. Prior to removal of the central disk portion, recipient embryos may optionally be exposed to irradiation (e.g. 490-680 rads of γ irradiation from a ^{60}Co source). After introduction of the donor germ-line competent cells into the recipient embryo, the recipient embryo is incubated to produce a chimeric embryo.

The recipient embryos containing the donor germ-line competent cells may be transferred preferably on the fourth day of development, into surrogate culture systems, to provide the appropriate environment to support development of a chicken embryo from syngamy to hatching. Suitable surrogate culture systems are described in Etches et al, (1996b).

The methods described herein improve the rate of transmission of the donor cell line to the recipient and provide larger numbers of chimeras. This facilitates commercial breeding programs to replace pure lines by genetically superior lines.

It will be appreciated that the methods of the present invention may be applied to other avian species including turkeys, ostriches, quail, pheasants, ducks, and geese.

IV. Identification of Germ-line Specific Molecules

The invention provides the isolation and identification of molecules (including genes and proteins) that are associated with germ-line cells. Such germ-line specific molecules can be used as markers to identify and separate germ-line cells from a population of cells using the methods of the present invention.

The germ-line specific molecules can be isolated using a variety of techniques known in the art. Methods to detect germ-line specific molecules include digestion of the germ-line cells with restriction endonucleases followed by analysis of the resulting fragments, differential hybridization of oligonucleotides, direct PCR sequencing, differential hybridization of oligonucleotides and denaturing gradient gel electrophoresis.

In one example, the presence of a germ-line specific molecule can be detected by the differential hybridization of oligonucleotides. In particular, germ-line cells can be isolated using the methods of the invention and the messenger RNA may be obtained from the cells. A cDNA library may be constructed from mRNA prepared from a pool of control cells. cDNA libraries may be synthetized using Oligo-dT primers and reverse transcriptase according to standard protocols. For example, cDNA may be cloned into plasmid vectors such as pSPORT R (Gibco BRL). Commercially available cDNA libraries may also be used such as phage display random libraries. The germ-line cell mRNA can be used to probe a library for sequences that specifically hybridize to the mRNA from the germ-line cells but n t to mRNA isolated from control cells.

Hybridization conditions which may be used in the methods of the invention are kn wn in the art and are described for example in Sambrook J, Fritch EF, Maniatis T. In:

Molecular Cloning, A Laboratory Manual, 1989. (Nolan C, Ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, which is incorporated herein by reference. The hybridization product may be assayed using techniques known in the art.

In another example, subtractive hybridization can also be used. Subtractive hybridization can identify and enrich genes that are differentially expressed in germ-line competent cells. Subtractive hybridization can be carried out by hybridizing between two DNA (or RNA) populations that are closely related such as the germ-line competent cells of the invention and other cells present in the stage X embryos. By subtractive hybridization, the hybridized sequences common to both cell types can be removed. Subsequently, the unhybridized sequences can be preserved as a subtracted cDNA library. Subtractive hybridization techniques known in the art can be used for example Christian E. Gruber and Wu-Bo Li, "An Improved Subtractive Hybridization Method using Phagemid Vectors", *Molecular Biology Current Innovations and Future Trends Part 1*, A.M: Griffen and H.G. Griffen (eds), 1995, which is incorporated herein by reference.

In another example, germ-line specific molecules could be detected using denaturing gradient gels. Restriction endonuclease fragments or PCR fragments associated with a selected nucleotide segment of germ-line specific cells can be resolved on a polyacrylamide gel containing gradients of denaturants, such as increasing temperature, increasing formamide, urea, and the like. The germ-line specific cell fragment and control fragments will denature at different positions in the gel leading to altered migration distances. The resolved fragments can the be detected by DNA hybridization for restriction fragments or direct DNA straining for PCR fragments.

Accordingly, the present invention provides a method of detecting germ-line specific molecules using any of the above methods.

Regulatory sequences such as promoters that control expression of the germ-line specific molecules may also be isolated.

Identification of germ-line specific molecules of the invention also permits the identification and isolation, or synthesis of nucleotide sequences which may be used as primers to amplify a nucleic acid molecule of the invention, for example in the polymerase chain reaction (PCR).

Accordingly, the present invention includes a method of determining the presence of a germ-line specific nucleic acid molecule of the invention is provided comprising treating the sample with primers which are capable of amplifying the nucleic acid molecule or a predetermined oligonucleotide fragment thereof in a polymerase chain reaction to form amplified sequences, under conditions which permit the formation of amplified sequences and, assaying for amplified sequences.

The polymerase chain reaction refers to a process for amplifying a target nucleic acid sequence as generally described in Innis et al, Academic Press, 1990 in Mullis el

al., U.S. Pat. No. 4,863,195 and Mullis, U.S. Patent No. 4,683,202 which are incorporated herein by reference. Conditions for amplifying a nucleic acid template are described in M.A. Innis and D.H. Gelfand, *PCR Protocols, A Guide to Methods and Applications* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White eds, pp3-12, Academic Press 1989, which is also incorporated herein by reference.

The amplified products can be isolated and distinguished based on their respective sizes using techniques known in the art. For example, after amplification, the DNA sample can be separated on an agarose gel and visualized, after staining with ethidium bromide, under ultra violet (UW) light. DNA may be amplified to a desired level and a further extension reaction may be performed to incorporate nucleotide derivatives having detectable markers such as radioactive labelled or biotin labelled nucleoside triphosphates. The primers may also be labelled with detectable markers as discussed above. The detectable markers may be analyzed by restriction and electrophoretic separation or other techniques known in the art.

The conditions which may be employed in the methods of the invention using PCR are those which permit hybridization and amplification reactions to proceed in the presence of DNA in a sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art. For example, see M.A. Innis and D.H. Gelfand, *PCR Protocols, A guide to Methods and Applications*, M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White eds, pp3-12, Academic Press 1989, which is incorporated herein by reference. Preferably, the PCR utilizes polymerase obtained from the thermophilic bacterium *Thermus aquatics* (Taq polymerase, GeneAmp Kit, Perkin Elmer Cetus) or other thermostable polymerase may be used to amplify DNA template strands.

It will be appreciated that other techniques such as the Ligase Chain Reaction (LCR) and NASBA may be used to amplify a nucleic acid molecule of the invention (Barney in "PCR Methods and Applications", August 1991, Vol.1(1), page 5, and European Published Application No. 0320308, published June 14, 1989, and U.S. Serial No. 5,130,238 to Malek).

Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of the expression of the nucleic acid molecules encoding a germ-line specific molecule of the invention. For example, RNA may be isolated from a cell type or tissue known to express a nucleic acid of the invention and tested utilizing the hybridization (e.g. standard Northern analyses) or PCR techniques referred to herein. The techniques may be used to detect differences in transcript size which may be due to normal or abnormal alternative splicing.

The isolated molecules may be used to prepare antibodies which can be used in the methods of the present invention to select germ-line competent cells. Conventional methods can be used to prepare the antibodies as described in detail above.

The following non-limiting example is illustrative of the present invention:

Example

The following materials and methods were used in the studies described in the Example:

Extirpation of germ-line competent cells from stage X embryos

Donor embryos were obtained from Barred Plymouth Rocks that are homozygous recessive (ii) at the I locus. Recipient embryos were obtained from White Leghorns that are homozygous dominant (II) at the I locus. The down colour of the Barred Plymouth Rocks and White Leghorns are black and yellow, respectively. These phenotypes facilitate determination of chimerism by feather colour when chicks hatch: chicks with black down were designated as somatic chimeras whereas yellow chicks were designated as putative chimeras. The contribution of donor- and recipient-derived cells to the germ-line was estimated by mating chimeras to Barred Plymouth Rocks and the extent of germ-line chimerism was expressed as the ratio of the number of Barred Rock to White Leghorn offspring.

Donor cells were obtained from Stage X blastoderms (Eyal-Giladi and Kochav, 1976) isolated from freshly laid, unincubated eggs as described by Carsience et al. (1993). Briefly, the albumen was separated from the yolk, the embryo was isolated, and the blastodermal cells were dispersed by digesting the extracelluar matrix with trypsin. The cells were then washed and resuspended in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum (FBS). Between 100 and 500 cells in 2-5 µl of medium were injected into the subgerminal cavity of recipient embryos.

Recipient embryos were physically compromised by removing a portion of the central disk or removing a portion of the lateral edge of the embryo, and control embryos were left intact. Approximately half of the embryos that were physically compromised and all of the control embryos were exposed to 490-680 rads of g irradiation from a ^{60}Co source within one hour after oviposition and within 2 h before they were physically compromised.

Donor cells were injected into all of the recipient embryos that were irradiated but not physically compromised and approximately one half of the recipient embryos that were physically compromised with or without exposure to irradiation. On the fourth day after injection, the embryos were transferred to surrogate shells and incubated t term as described by Etches et al. (1996b).

Preparation of Culture Dishes

SNL 76/7 murine fibroblast feeder cells (a gift from A. Bradley, Baylor College of Medicine, Houston, TX, USA) were seeded on 0.1% sterile bovine skin gelatin (Sigma, St. Louis, MO, USA) coated culture dishes (Nunc, Denmark) at a density of 1.25 x 10^5 cells/cm^2. Feeder cells were maintained in Dulbecco's modified Eagle's medium (DMEM)(Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Cansera, Rexdale, ON, Canada), 2mM L-glutamine (Gibco), 100 IU/ml penicillin and 100µg/ml streptomycin (Gibco) at pH 7.2 for 24 hours at 37°C in 5% CO_2.

Dissociation of Cells

Stage X blastoderms were isolated from Barred Plymouth Rock embryos into a Petri dish in Dulbecco's phosphate buffered saline containing 1 g/L D-glucose (PBS-G) using sterile filter paper rings (Petitte et al., 1990). The entire blastoderm was carefully cleaned of excess yolk and gently transferred with a Pasteur pipette into a 15 ml centrifuge tube containing 2 to 3 ml of PBS-G plus 2% (v/v) chicken serum (ChS). Blastoderms were pooled at two per ml. The PBS-G + 2% ChS was removed and replaced with an equal volume of calcium and magnesium free phosphate buffered saline (CMF-PBS) + 2% ChS. Blastoderms were then incubated for 10 minutes on ice. The CMF-PBS was replaced with 0.05% trypsin (w/v) in 0.02% EDTA (w/v) (Gibco) and blastoderms were again incubated for 10 minutes on ice. Trypsin was replaced with 1.0 ml of Opt-modified Eagle's medium I (Optimem) (Gibco) supplemented with 2% ChS, 4% (v/v) FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin at pH 7.2. The blastoderms were pooled and slowly dispersed by aspiration with a 10 ml pipette. A sample of cell suspension was used to determine cell concentration with a haemocytometer. Dissociated cells were centrifuged for 4 minutes at 1000 rpm. The supernatant was removed and cells were resuspended in an appropriate volume of Optimem. Dispersed blastodermal cells were plated on SNL feeder cells at a concentration of $1x10^5$ cells/cm^2 incubated for 1, 3, 7, or 18 hours at 37°C in 5% CO_2.

Determination of Immunohistochemistry

Cultured blastodermal cells and whole mounts were fixed in 4% paraformaldehyde (Sigma) for 10 minutes at 4°C. The preparations were then immersed for 4 hours in PBS supplemented with 1 mg/ml bovine serum albumin (PBS-BSA) (Boehringer Mannheim, Laval, PQ, Canada), plus 5% (v/v) goat serum (Sigma) at 4°C to saturate non-specific binding sites. The blocking solution was replaced with supernatant from SSEA-1 diluted 1:3 in PBS-BSA, EMA-1 diluted 1:10 in PBS-BSA or HNK-1/NC-1 (a gift from C. Stern, Columbia University, NY, USA) diluted 1:2 at 4°C with gentle rocking for at least 12 hours. SSEA-1 and EMA-1 antibody supernatants were obtained fr m the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacol gy and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD, and the Department

of Biological Sciences, University of Iowa, Iowa City, IA under contract N01-HD-6-2915 from the NICHD. The preparations were washed two times in PBS-BSA and incubated for 2 hours at 4°C in the dark with affinity-purified goat anti-mouse IgM-FITC conjugate diluted 1:50 (Jackson ImmunoResearch, West Grove, PA, USA). After washing twice with PBS-BSA, the preparations were mounted with glycerol diluted 1:1 with PBS and viewed on a Nikon Diaphot-TMD inverted microscope equipped with epifluorescent optics, excited by a mercury lamp, using filters to allow emission at 470-490 nm.

Selection of cells using magnetic beads

Dissociated cell preparations were incubated with monoclonal antibodies SSEA-1, EMA-1 or NC-1 (Immunotech, Westbrook, ME, USA) for 45 minutes at 4°C. Before incubation, blastodermal cells were isolated as outlined above, except that trypsin was replaced with PBS-ChS. Subsequently, the cell preparation was washed in PBS-BSA and incubated with 100 µl of PBS-BSA containing rat anti-mouse IgM conjugated microbeads (Miltenyi Biotec, Sunnyvale, CA, USA) for 30 minutes at 4°C. The mixture was then washed again and resuspended in 500 µl of PBS-BSA. A steelwool separation column (type MS+: Miltenyi Biotech) was inserted into a MiniMACS magnetic system (Miltenyi Biotech) and rinsed with PBS-BSA. The cell suspension was run through the column. Unbound (negative) cells were flushed out with PBS-BSA. Cells labelled with the superparamagnetic beads are magnetic in a magnetic field and bind to the steelwool fibers of the column, while unlabelled cells pass through the column (Miltenyi et al., 1990). Finally, the column was removed from the magnetic field and the bound (positive) cells eluted by rinsing with PBS-BSA. Immunohistochemistry was performed on the cells before and after magnetic cell sorting.

Results:

The reproductive capacity of birds derived from embryos in which the central and lateral regions were extirpated with and without irradiation are shown in Tables 1 and 2. It is evident that production of eggs and sperm and that the fertilizing capacity of the gametes were unaffected by the method of treating embryos. It can be concluded, therefore, that any residual germ-line competent cells remaining after extirpation of the central region had the capacity to proliferate and populate the germ-line with a normal complement of spermatogonia in males and oogonia in females.

The number of somatic and germ-line chimeras that were produced after injection of Barred Plymouth Rock donor cells into White Leghorn recipient embryos that were compromised by irradiation and extirpation of approximately 500 cells from the central or lateral regions of the embryos are shown in Table 3-6. Somatic chimeras hatch with some black (donor-derived) pigmentation (see column 2 in Table 3-6). Putative chimeras have no black (donor-derived) pigmentation. Previous work from our laboratory has shown that putative chimeras usually are not germ-line chimeras. When embryos were

irradiated and approximately 500 cells were injected into the central regions of the embryo (Table 3), 5 of the 9 somatic chimeras produced donor-derived offspring, (i.e. they were germ-line chimeras). By comparison only 1 of 8 somatic chimeras produced by injecting 500 cells into the lateral region of irradiated recipient embryos (Table 4) produced donor-derived offspring. These data are consistent with and lend support to the proposal that germ-line competent cells are located in the center of the stage X embryo.

Injection of Barred Plymouth Rock donor cells into the central region of recipient embryos that had been compromised by extirpating approximately 500 cells from the center of the embryo yielded 3 germ-line chimeras from 9 somatic chimeras (Table 5). By comparison, injection of Barred Plymouth Rock cells into the lateral region of recipients from which cells had been removed from that lateral region yielded only 1 germ-line chimera among 10 somatic chimeras (Table 6). These data are also consistent with the hypothesis that germ-line competent cells reside in the central region of the embryo. In addition, the rate of germ-line chimerism produced following compromising recipient embryos by irradiation and by physically removing cells from the central region are similar. Irradiation of recipient embryos is believed to improve the rate of germ-line chimerism by slowing the rate of development of the recipient embryo. Since the donor cells have not been irradiated, they proliferate while cell division is inhibited in the recipient embryo and consequently, the ratio of donor recipient cells in the chimera is increased (Carsience et al., 1993). Removal of cells from the center of the embryo also increases the ratio of germ-line chimerism (from 0.6% to 11.3%, see Table 7). In this case, the increase is attributed to the removal of germ-line cells from the center of the recipient and the subsequent reintroduction of donor germ-line competent cells to the same area. This interpretation is supported by the very low rate of germ-line transmission that follows removal of cells from the lateral regions of the embryo (Table 7).

Identification of germ-line competent cells by immunofluorescence detection of cells expressing epitopes

Identification of Cells Expressing Epitopes by Immunofluorescence

Labelling of cells expressing surface antigens recognized by EMA-1 and SSEA-1 could be detected in stage X Barred Plymouth Rock whole embryos (Figures 2 and 3). A mixture of non-, weakly and highly fluorescent staining cells were observed after 1, 3, 7 and 18 hours in culture. Approximately 8%, 15% and 48% of cells cultured for 18 hours showed intense staining for EMA-1 (Figures 4 and 5), SSEA-1 (Figures 6 and 7), and HNK-1/NC-1 (Figures 8 and 9), respectively. Cultures of SNL murine fibroblast cells were used as negative controls. Reagent controls consisted of replacing the primary antibody with PBS-BSA. None of the cells in the negative and reagent controls were unstained.

Selection of cells expressing epitopes using magnetic beads

A three fold enrichment of Stage-X blastodermal cells expressing the SSEA-1 epitope was achieved using SSEA-1 coated microbeads. Immunofluorescence verified that the percentage of SSEA-1 staining cells increased from 14% to 45% after separation. Immunomagnetic isolation of EMA-1 positive cells resulted in a three fold enrichment from 8% of the cells before purification to 28% after purification. Selection for cells expressing NC-1 epitopes resulted in a two fold increase. The percentage of NC-1 positive cells increased from 48% to 89% after separation. In all cases, viability of cells after separation was found to be greater that 94% as determined by trypan blue exclusion.

The rates of somatic and germ-line chimerism and the rate of germ-line transmission of male and female chimeras made using the positive and negative fractions from MACS columns containing EMA-1, SSEA-1 and NC-1 are presented in Table 8 and 9 respectively. The overall rates of both somatic and germ-line chimersim were low in these experiments, indicating that manipulation of the cells in the magnetic columns reduced their overall ability to contribute to recipient embryos. Preliminary evidence from these experiments indicates that the contribution to somatic and germ-line tissues of the positive and negative fractions selected using SSEA-1 and NC-1 were approximately equal. Cells that were selected by the EMA-1 antibody, however, yielded significant contributions to the germ-line in 2 of 3 chimeras (Table 8). These data indicate that germ-line competent cells can be selected using the EMA-1 epitope. Since the enrichment of EMA-1 positive cells using MACS is from 8% to 28%, (see above), approximately 72% of the cells that were injected were not EMA-1 positive.

In summary, these data support the conclusion that a population of germ-line competent cells exists in the central region of the stage X (E-G&K) embryo and that the germ-line competent cells can be isolated using a technique such as magnetically activated cell sorting using antibodies such as EMA-1 that recognize epitopes that are specifically expressed by germ-line competent cells.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification and detailed legends for the figures are provided.

Table 1. Mean age and the range in age at the onset of egg production and fertility of hens derived from Stage X embryos that were irradiated or from which approximately 500 cells were extirpated.

Irradiation	Lateral removal of cells	Central removal of cells	Number of hens	Mean age at onset of lay	Range in age at the onset of lay	Mean % Fertility (100 x number of chicks hatched/number of eggs set) and range in parentheses
+	+	-	2	151	142-160	89 (88-90)
+	-	+	3	161	151-167	84 (75-98)
-	+	-	6	155	139-177	83 (74-90)
-	-	+	4	139	117-158	80 (77-82)

Table 2. Mean concentration of spermatozoa and fertility of males derived from Stage X embryos that were irradiated or from which approximately 500 cells were extirpated.

Irradiation	Lateral removal of cells	Central removal of cells	Number of males	Mean concentration (10^9 cells/ml) of spermatozoa and range in parentheses	Mean % Fertility (100 x number of chicks hatched/number of eggs set) and range in parentheses
+	+	-	3	5.5 (4.9 - 5.9)	75 (66 - 91)
+	-	+	2	6.6 (5.0 - 8.2)	67 (65 - 69)
-	+	-	3	5.2 (3.3 - 8.2)	71 (57 - 81)
-	-	+	4	3.6 (0.9 - 5.6)	66 (57 - 71)

Table 3. The number of recipient- and donor-derived offspring from test mating somatic and putative chimeras to Barred Plymouth rocks. Group 1 - Recipient embryos compromised by irradiation and injected with ~500 donor cells centrally.

Description of Chimera	Percentage of black pigmentation	Number of donor-derived offspring	Number of recipient-derived offspring	Percentage of donor-derived offspring
Somatic female				
2583-2584	90	0	135	0
2577-2578	1	0	100	0
2589-2590	75	0	116	0
2324-2325	95	43	38	53.1
2327-2328	99	3	14	17.6
Somatic males				
2575-2576	95	106	220	32.5
2579-2580	80	0	302	0
2581-2582	80	69	205	25.2
2329-2330	20	31	102	23.3

Table 4. The number of recipient-and donor-derived offspring from test mating somatic and putative chimeras to Barred Plymouth rocks. Group 2 - Recipient embryos compromised by irradiation and injected with -500 cells laterally.

Description of Chimera	Percentage of black pigmentation	Number of donor-derived offspring	Number of recipient-derived offspring	Percentage of donor-derived offspring
Somatic female				
2401-2402	99	0	0	0
2430-2431	2	29	154	15.8
2466-2467	30	0	139	0
2468-2469	95	0	127	0
2370-2471	95	0	1	0
Somatic males				
2425-2526	75	0	312	0
2436-2437	20	0	297	0
2472-2473	40	0	240	0

Table 5. The number of recipient-and donor-derived offspring from test mating somatic and putative chimeras to Barred Plymouth rocks. Group 3 - Recipient embryos compromised by physically removing -500 cells from the central area and injected with -500 donor cells centrally.

Description of Chimera	Percentage of black pigmentation	Number of donor-derived offspring	Number of recipient-derived offspring	Percentage of donor-derived offspring
Somatic female				
2499-2500	80	0	91	0
2531-2532	50	0	57	0
2533-2534	40	1	72	1.4
2591-2592	20	0	1	0
2593-2594	20	0	111	0
2347-2348	2	0	108	0
Somatic males				
2505-2506	50	63	191	24.8
2524-2525	45	24	270	8.1
2345-2346	1	0	159	0

Table 6. The number of recipient - donor-derived offspring from test mating somatic and putative chimeras to Barred Plymouth rocks. Group 4 - Recipient embryos compromised by physically removing -500 cells form the lateral area and injected with -500 donor cells laterally.

Description of Chimera	Percentage of black pigmentation	Number of donor-derived offspring	Number of recipient-derived offspring	Percentage of donor-derived offspring
Somatic female				
2409-2410	20	1	177	0.6
2415-2416	2	0	160	0
2305-2427	20	0	138	0
2444-2445	40	0	133	0
2448-2450	10	0	84	0
2460-2308	20	0	129	0
2474-2475	75	0	124	0
Somatic males				
2417-2418	1	0	378	0
2419-2429	35	0	377	0
2456-2457	50	0	316	0

Table 7. Rates of somatic chimerism expressed as the % of black pigmentation in plumage and the rate of germ-line transmission expressed as the number of donor-derived offspring/total number of offspring from chimeras derived from Stage X embryos that were irradiated or from which approximately 500 cells were extirpated.

Irradiation	Lateral removal of cells	Central removal of cells	Number of somatic chimeras	Somatic chimerism (% black pigmentation)	Number of germ-line chimeras	Rate of germ-line transmission (number of donor-derived offspring/total number of offspring x 100)
+	+	-	8	57	1	15.8
+	-	+	9	71	5	30.3
-	+	-	10	27	1	0.6
-	-	+	9	34	3	11.3

Table 8. Proportion of male somatic and germ-line chimeras made by injecting cells selected using EMA-1, SSEA-1 and NC-1 epitopes by magnetic activated cells sorting (MACS).

Ab	Fraction	% somatic chimerism	Number of germ-line chimeras/number of chimeras	Rate of germ-line chimerism (%)
EMA-1	+	40	2/3	17
	-	10	0/1	–
SSEA-1	+	24	1/4	5.5
	-	44	4/10	6.9
NC-1	+	57	5/10	22.0
	-	52	3/6	13.4

Table 9. Proportion of female somatic and germ-line chimeras made by injecting cells selected using EMA-1, SSEA-1 and NC-1 epitopes by magnetic activated cells sorting (MACS).

Ab	Fraction	% somatic chimerism	Number of germ-line chimeras/number of chimeras	Rate of germ-line chimerism (%)
EMA-1	+	52	0/3	–
	-	85	0/1	–
SSEA-1	+	45	2/7	3.2
	-	54	3/11	4.5
NC-1	+	44	1/11	36
	-	42	0/10	–

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DETAILED FIGURE LEGENDS

Figure 1. The structure of an embryo at the time of laying illustrated as a cross-section through the embryo perpendicular to the surface of the yolk. At this time, the embryo contains 40,000-60,000 cells and is referred to as a Stage X embryo.

Figure 2. Phase contrast illumination micrograph of a microscopic field illustrating the expression of EMA-1 epitopes on stage X blastodermal cells cultured 18 hours with SNL feeder cells (magnification 300x).

Figure 3. Fluorescence micrograph of the same microscopic field as figure 2 illustrating the expression of EMA-1 epitopes on stage X blastodermal cells cultured 18 hours with SNL feeder cells (magnification 300x).

Figure 4. Phase contrast illumination micrograph a microscopic field illustrating the expression of SSEA-1 epitopes after 18 hours in culture.

Figure 5. Fluorescence micrograph of the same microscopic field as figure 4 illustrating the expression of SSEA-1 epitopes after 18 hours in culture. Note the variation in fluorescence intensity.

Figure 6. Phase contrast illumination micrograph of a microscopic field illustrating the SSEA-1 expression on stage X whole mount (magnification 300X).

Figure 7. Fluorescence micrographs of the same microscopic field as figure 1 illustrating the SSEA-1 expression on stage X whole mount (magnification 300X).

Figure 8. Phase contrast illumination micrograph of a microscopic field illustrating HNK-1/NC-1 expression on stage X blastodermal cells after 18 hours in culture.

Figure 9. Fluorescent micrograph of the same microscopic field as figure 8 illustrating HNK-1/NC-1 expression on stage X blastodermal cells after 18 hours in culture.

I Claim:

1. A method for selecting germ-line competent cells in stage X (E-G&K) chicken embryos comprising separating from a stage X (E-G&K) chicken embryo cells that have an epitope expressed by germ-line competent cells associated with their cell surface.

2. A method according to claim 1 comprising reacting the chicken embryo cells with a substance that binds to the epitope; forming conjugates between the substance and the germ-line competent cells having the epitope associated with their cell surface; and isolating the conjugates to obtain a cell preparation containing germ-line competent cells.

3. A method according to claim 2 wherein the substance that binds to the epitope is an antibody.

4. A method according to claim 3 wherein the antibodies are conjugated, either directly or indirectly, to magnetic beads.

5. A method according to claim 1 wherein the epitope is EMA-1.

6. A method according to claim 1 wherein the epitope is SSEA-1.

7. A method for producing a chimeric chicken embryo comprising (a) providing germ-line competent cells isolated from a stage X (E-G&K) chicken embryo; (b) introducing the germ-line competent cells into a recipient stage X (E-G&K) chicken embryo from which a portion of the central disk of the embryo has been removed; and (c) incubating the recipient embryo to produce a chimeric embryo.

8. A method according to claim 7 wherein the germ-line competent cells are isolated by reacting stage X (E-G&K) chicken embryo cells with a substance that binds to an epitope expressed by germ-line competent cells; forming conjugates between the substance and the germ-line competent cells having the epitope associated with their cell surface; and removing the conjugates to isolate a cell preparation containing germ-line competent cells.

9. A method according to claim 7 further comprising transfecting the germ-line competent cells with a recombinant expression vector containing an exogenous gene and the necessary elements for the transcription and translati n of the gene prior to introducing the cells int the recipient.

10. A method according to claim 7 wherein the substance that binds to the epitope is an antibody.

11. A method according to claim 7 wherein the epitope is EMA-1.

12. A method according to claim 7 wherein the epitope is SSEA-1.

13. A method according to claim 7 wherein the central disk portion of the recipient embryo is removed using physical techniques.

14. A method according to claim 7 wherein the central disk portion of the recipient embryo is removed using an antibody that binds to germ-line competent cells coupled to a toxin.

15. A method according to claim 7 wherein the recipient embryo is irradiated prior to removal of the central disk portion.

16. A method for identifying a region in an embryo which contains germ-line competent cells comprising:

(a) treating the embryo with a substance which directly or indirectly binds to an epitope expressed by germ-line competent cells wherein the substance is labelled with a detectable marker; and

(b) detecting the detectable change produced by the detectable marker to identify a region in the embryo which contains germ-line competent cells.

17. A method according to claim 16 wherein the substance is an antibody.

18. A method according to claim 16 wherein the epitope is EMA-1.

19. A method according to claim 16 wherein the epitope is SSEA-1.

FIGURE 1

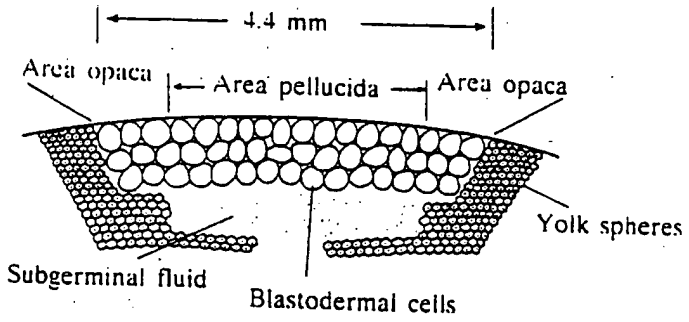
4.4 mm
Area opaca
Area pellucida
Area opaca
Yolk spheres
Subgerminal fluid
Blastodermal cells

FIGURE 2

FIGURE 3

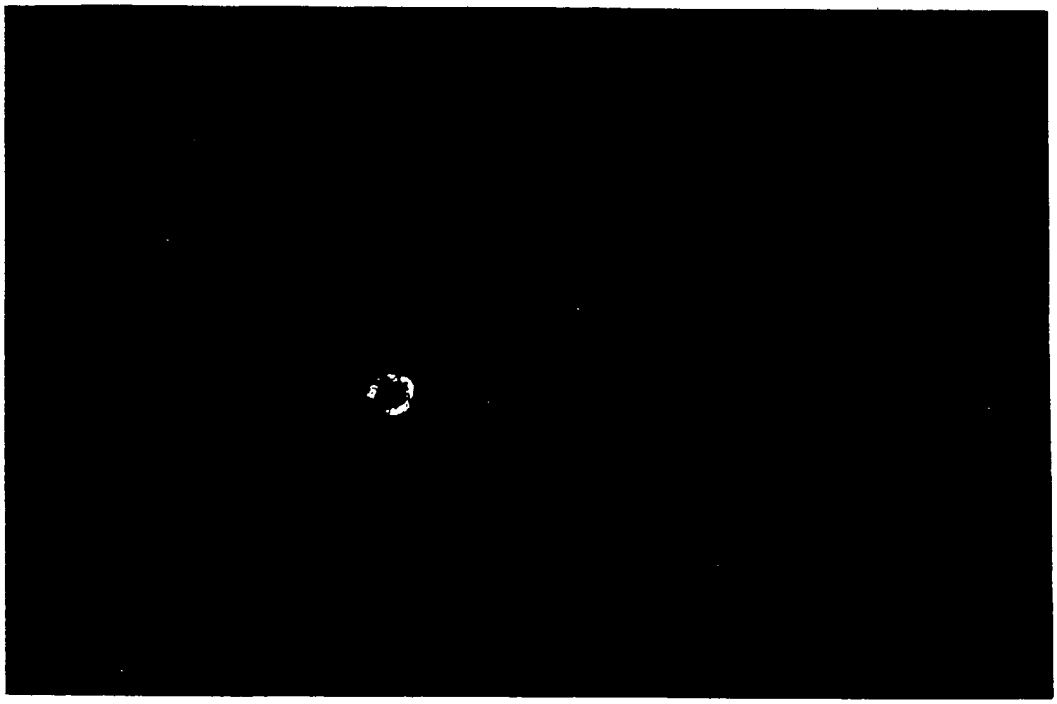

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FIGURE 4

FIGURE 5

FIGURE 6

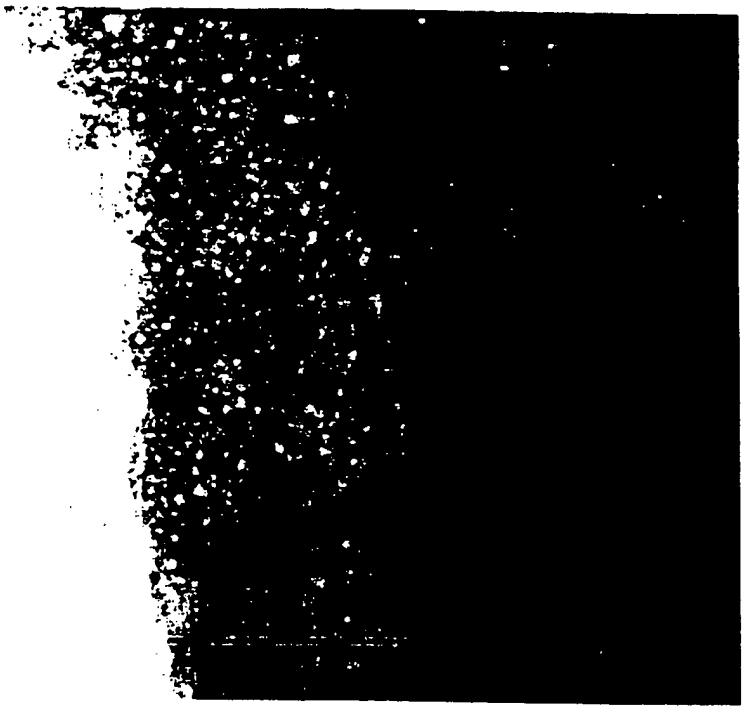

FIGURE 7

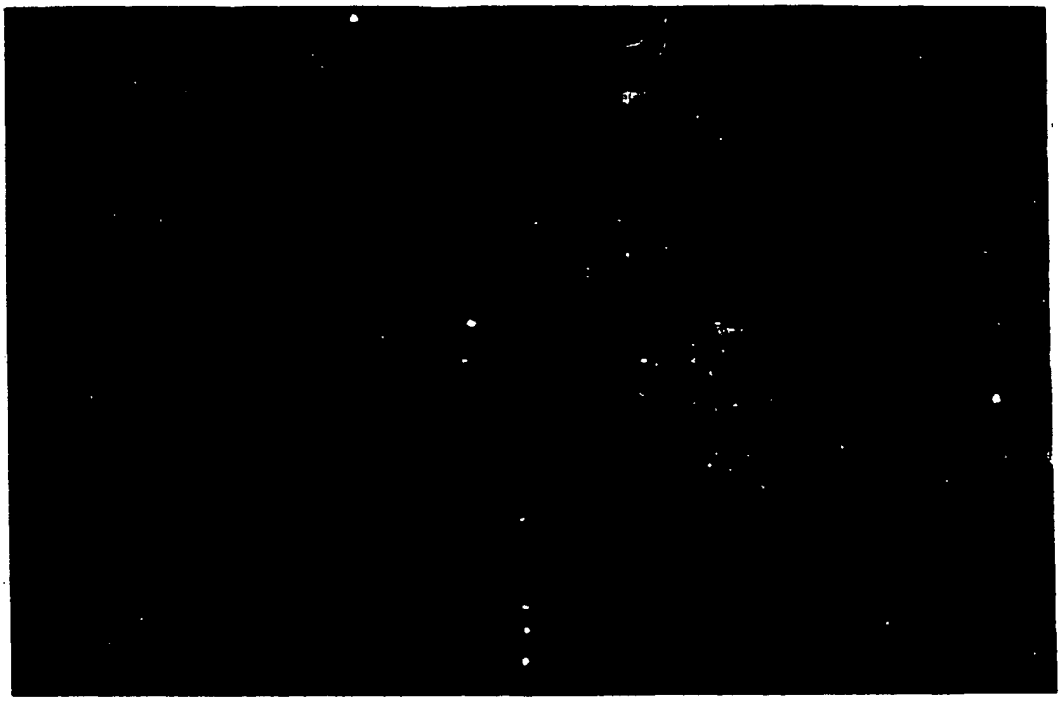

FIGURE 8

FIGURE 9

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/CA 98/00145

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 A01K67/027

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PETITTE, J.N. & KARAGENÇ, L.: "growth factors during early events in avian embryo development" POULTRY AND AVIAN BIOLOGY REVIEWS, vol. 7, no. 2/3, 1996, pages 75-87, XP002069537 cited in the application * pages 80; page 81, left-hand column *	1-8, 10-13, 16-19
X Y	EP 0 710 439 A (710439) 8 May 1996 * p. 2, lines 26 - 34; p. 3, lines 24-26 *	1 1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 June 1998	23/07/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/CA 98/00145

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KARAGENÇ, L. ET AL.: "Origin of primordial germ cells in the prestreak chick embryo" DEVELOPMENTAL GENETICS, vol. 19, 1996, pages 290-301, XP002069538 * whole disclosure *	1-19
Y	ETCHES, R.J. ET AL.: "Chimeric chickens and their use in manipulation of the chicken genome" POULTRY SCIENCE, vol. 72, 1993, pages 882-889, XP002069539 cited in the application * figure 1 *	9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. onal Application No - PCT/CA 98/00145

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 710439 A	08-05-1996	JP 2535792 B	18-09-1996
		JP 8127501 A	21-05-1996
		US 5759763 A	02-06-1998